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RESEARCH ARTICLE

Microscale genetic differentiation in a sessile invertebrate with cloned larvae: investigating the role of polyembryony

Andrew J. Pemberton · Lars J. Hansson · Sean F. Craig · Roger N. Hughes · John D. D. Bishop

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Abstract Microscale genetic differentiation of sessile organisms can arise from restricted dispersal of sexual propagules, leading to isolation by distance, or from localised cloning. Cyclostome bryozoans offer a possible combination of both: the localised transfer of spermatozoa between mates with limited dispersal of the resulting larvae, in association with the splitting of each sexually produced embryo into many clonal copies (polyembryony). We spatially sampled 157 colonies of *Crisia denticulata* from subtidal rock overhangs from one shore in Devon, England at a geographic scale of ca. 0.05 to 130 m plus a further 21 colonies from Pembrokeshire, Wales as an outgroup. Analysis of molecular variance (AMOVA) revealed that the majority (67%) of genetic variation was distributed among individuals within single rock overhangs, with only 16% of variation among different overhangs within each shore and 17% of variation between the ingroup and outgroup shores. Despite local genetic variation, pairwise genetic similarity analysed by spatial autocorrelation was greatest at the smallest inter-individual distance we tested (5 cm) and remained significant and positive across generally within-overhang comparisons (<4 m). Spatial autocorrelation and AMOVA analyses both indicated that patches of *C. denticulata* located on different rock overhangs tended to be genetically distinct, with the switch from positive to negative autocorrelation, which is often considered to be the distance within which individuals reproduce with their close relatives or the radius of a patch, occurring at the 4–8 m distance class. Rerunning analyses with twenty data sets that only included one individual of each multilocus genotype ($n = 97$) or the single data set that contained just the unique genotypes ($n = 67$) revealed that the presence of repeat genotypes had an impact on genetic structuring (PhiPT values were reduced when shared genotypes were removed from the dataset) but that it was not great and only statistically evident at distances between individuals of 1–2 m. Comparisons to a further 20 randomisations of the data set that were performed irrespective of genotype ($n = 97$) suggested that this conclusion is not an artefact of reduced sample size. A resampling procedure using kinship coefficients, implemented by the software package GENCLONE gave broadly similar results but the greater statistical power allowed small but significant impacts of repeat

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A. J. Pemberton · L. J. Hansson · J. D. D. Bishop
The Laboratory, Marine Biological Association of the U.K, Citadel Hill, Plymouth PL1 2PB, UK

S. F. Craig · R. N. Hughes
School of Biological Sciences, University of Wales, Bangor, Gwynedd LL57 2UW, UK

J. D. D. Bishop
School of Biological Sciences, University of Plymouth, Drake Circus, Plymouth PL4 8AA, UK

A. J. Pemberton (✉)
Zoological Museum of the University of Zurich, Winterthurerstrasse 190, 8057 Zurich, Switzerland
e-mail: andrew.pemberton@gmail.com

Present Address:
L. J. Hansson
SMHI, Nya Varvet 31, 426 71 Västra Frölunda, Sweden

Present Address:
S. F. Craig
Department of Biological Sciences, Humboldt State University, Arcata CA 95521, USA

genotypes on genetic structure to be also detected at 0.125–0.5 and 4–16 m. Although we predict that a proportion of the repeat multilocus genotypes are shared by chance, such generally within-overhang distances may represent a common distance of cloned larval dispersal. These results suggests that closely situated potential mates include a significant proportion of the available genetic diversity within a population, making it unlikely that, as previously hypothesised, the potential disadvantage of producing clonal broods through polyembryony is offset by genetic uniformity within the mating neighbourhood. We also report an error in the published primer note of Craig et al. (Mol Ecol Notes 1:281–282, 2001): loci Cd5 and Cd6 appear to be the same microsatellite.

Introduction

Forces responsible for the evolution and maintenance of sexual reproduction remain enigmatic. The well documented problem is that sex should be prohibitively expensive compared to asexual reproduction. Most attention has been given to obligate sexuals or asexuals, but in many taxa sexual and asexual reproduction coexist (Bell 1982; Hughes 1989). The question then becomes one of when and how much sex is optimal and it appears that in many environments the best strategy may be to produce a small fraction of offspring sexually, and the rest asexually (Hurst and Peck 1996). One intriguing life history strategy that appears to depart from the optimal balance of sexual and asexual reproduction is polyembryony. 'Polyembryony' refers to the splitting of a single sexually produced embryo into many clonal copies. By incorporating a sexual phase the various costs of sex seem to be incurred. By then cloning, at least some of the advantages appear forgone. Producing polyembryonic offspring forsakes the genetic diversity inherent within a sexual brood and 'bets' on a single unproved genotype—an error equivalent to that of purchasing multiple tickets of the same number in a lottery (Craig et al. 1997). Despite such an apparent handicap, routine polyembryony (as compared to the occasional aberrant production of identical twins) appears to have evolved numerous times, being reported in some rust fungi, algae, higher plants and animals (metazoan phyla include cnidarians, platyhelminths, arthropods, bryozoans, echinoderms and chordates—see Craig et al. 1997; Hughes et al. 2005). More examples of embryonic cloning are being discovered as groups receive sufficient study (e.g. Eaves and Palmer 2003).

This work focuses on one order of bryozoans, the Cyclostomata (class Stenolaemata), in which all representatives (except the Cinctiporidae, Boardman et al. 1992) are thought to be polyembryonic (Ryland 1970). Although several circumstances have been suggested that should favour the evolution and maintenance of polyembryony (see Craig et al. 1995, 1997; Giron et al. 2004), most do not seem relevant to sessile organisms like cyclostome bryozoans that brood their offspring to a late stage of development (discussed further in S1.1 of the 'Electronic Supplementary Material'; hereafter ESM). In this paper we attempt to address a hypothesis for the maintenance of polyembryony in sessile organisms like cyclostome bryozoans made by Ryland (1996). Ryland predicted that the potential for cyclostome bryozoans to produce a genetically diverse sexual brood would be limited within the viscous population structures that would be expected to result from restricted gene flow in populations of colonial marine invertebrates. If all available spermatozoa originate from a similar genetic background the relative loss of offspring diversity caused by polyembryony is reduced. Investigation of the microgeographic genetic composition of a natural population of a cyclostome bryozoan could test this hypothesis. A finding that mating neighbourhoods were genetically homogeneous would support Ryland's proposition. Alternatively, evidence that significant genetic variation was distributed among neighbouring sperm donors would go against the hypothesis. As well as helping us to understand Ryland's (1996) proposition, information on genetic differentiation over small spatial scales may also allow us to quantify the impact of repeat genotypes, which may be the signature of multiple settlement of polyembryonous clonemates, on the genetic structure of the population in order to determine the relative contributions of restricted sperm and larval dispersal and polyembryony to any observed fine-scale genetic structuring.

The ability to use dilute sperm efficiently, and thus the potential for fertilisation at a distance, would have important consequences for gene flow, and therefore genetic structuring, in 'spermcast' or 'egg-brooding free-spawning' taxa that release sperm into the water but retain their eggs for internal fertilisation. Recent work (Bishop 1998; Yund 2000; Pemberton et al. 2003; Johnson and Yund 2004; Lasker 2006) published after Ryland's (1996) paper has demonstrated that the dynamics of fertilization in spermcast taxa may differ greatly from the externally fertilising models that rely on dispersing clouds of eggs and sperm intersecting during a crucial interval following spawning. Given their ability to accumulate sperm from low concentrations, cross fertilisation, from distances potentially sufficient to maintain genetic heterogeneity, appears to be obtainable in spermcast animals (Pemberton et al. 2003; Phillippi et al. 2004; but see S1.2 in the ESM for possible risks of extrapolating fertilisation data across taxa).

Colonial marine invertebrates typically possess non-feeding, lecithotrophic, larvae that disperse relatively short distances (Jackson 1986). Direct observation that larvae of

the cyclostome *Tubulipora tuba* were observed to settle on the same piece of kelp as their brood parent at densities ten times greater than on similar substrates only 1 m away (McFadden, personal communication in Knowlton and Jackson 1993) and indirect supposition from incidences of colony fusion of what are assumed to be polyembryonous clonemates of an extant cyclostome (Harmelin 1974) and an extinct fenestrate relative (McKinney 1981) support the idea of limited dispersal in cyclostome larvae. Limited dispersal can lead to genetic structuring of a population as a result of isolation-by-distance, with differentiation possible through genetic drift or local selection. However despite theoretical expectations of micro-scale genetic structuring (Knowlton and Jackson 1993) evidence of this exists for relatively few sessile invertebrates (Yund and O'Neil 2000). When found population subdivision may (e.g. Hellberg 1995) or may not (e.g. Burnett et al. 1995) be a result of the limited dispersal of larvae.

As asexual fragmentation and rafting of the adult stage is not expected in cyclostome bryozoans with the colony form and habitat of the species studied here (discussed in S1.3 in the ESM), the multiple settlement of genetically identical larvae cloned through polyembryony is the only route for asexual processes to influence microscale genetic differentiation. A number of previous studies have investigated the population genetic consequences of cloning at the adult stage in sessile marine invertebrates (e.g. McFadden 1997 and references therein) and algae (e.g. Wright et al. 2000; Hämmerli and Reusch 2003; Alberto et al. 2005). The population genetic consequences of cloning meiotically produced embryos are less well understood (Engel et al. 2004).

Materials and methods

Bryozoan biology

Bryozoans are aquatic invertebrates that are typically colonial and hermaphroditic, built from replicated zooids budded from a metamorphosed, sexually produced larva. Zooids filter feed with a ciliated, tentacular lophophore. Adjacent zooids are connected, allowing resources to be shared around the colony. Cyclostomes are polymorphic with, amongst others, distinct feeding (auto-) and brooding (gono-) zooids. Spermatogenesis in such species without dedicated male zooids occurs in autozooids, with sperm released into the water column (Silén 1972). In most bryozoan species eggs are retained and fertilisation is internal. In the class Gymnolaemata sperm are brought into contact with acting female zooids through the feeding current, adhere to tentacles on the lophophore, and enter the maternal coelom (Temkin 1994). Sperm collection in the Cyclostomata has not been described. Oogenesis in cyclostome bryozoans has been best studied within the genus *Crisia*, where zooidal development is closely linked to the process of branch formation from apical growing points within the erect colony. Adjacent zooids are calcified together into short lengths called internodes, connected by flexible nodes. Many oogonia initially form in the developing internode but most degrade, their host zooids differentiating into autozooids. On some internodes a single (rarely >1) developing zooid differentiates into a gonozooid, on which the lophophore is transitory. The calcareous wall of the developing gonozooid is expanded. Within this brooding space polyembryony occurs when a 'primary embryo' repeatedly buds off clumps of cells that, with slight variation between taxa, go on to develop into independent larvae (Harmer 1893; Robertson 1903; Borg 1926, Ryland 2000). A brood size of up to 100 embryos has been reported, although in *Crisia* embryos appear to develop, and are presumably released, sequentially such that at any one time the gonozooid contains many fewer mature larvae. Molecular techniques have recently confirmed that embryos within individual gonozooids of *Crisia denticulata* are genetically identical, but that different gonozooids on the same colony may be fertilised by different males (Hughes et al. 2005). Whether this is the case for all cyclostome species is unknown as other cyclostome families such as the Lichenoporidae or Diastoporidae, which await molecular analysis, have single or fused gonozooids. The Hughes et al. (2005) data found no evidence for self-fertilization. The concurrent retention of genetic diversity between broods is common in non-animal, sessile, polyembryonous taxa such as pines (e.g. Krutovskii and Politov 1995; Filonova et al. 2002) and red algae (e.g. Engel et al. 2004).

Sample collection and genotyping

Colonies of *Crisia denticulata* were mapped and collected from the underside of rock overhangs in the shallow subtidal by snorkelling at low water springs. Overhangs extended ca. 1–6 m horizontally along the shore and projected ca. 0.5–1.5 m from top to bottom as measured along the rock surface. Each overhang, which may have contained one colony to several hundred colonies, was separated horizontally from adjacent overhangs by ca. 2–30 m. Density of *C. denticulata* across all sampled overhangs was mapped semi-quantitatively within a grid held against the rock wall (5 × 5 cm grid divisions: score 0 = no colonies present; 1 = one or two colonies; 2 = many colonies, >50% cover of *C. denticulata*). The position of each collected colony was recorded from its *x*,*y* position within the grid. Colonies were collected semi-randomly to ensure representation from across the range of local densities. Samples from Wembury, near Plymouth, Devon, England (hereafter 'Wembury') were collected over the summer of 2001 and

are an extension of those first reported by Hughes et al. (2005). Aerial photographs of the study site are provided in S2 in the ESM along with a summary of all mapping, density and genotyping data. Samples from Caerfai Bay, near St Davids, Pembrokeshire, Wales (hereafter 'Pembrokeshire', national grid reference SM762241) were collected in July, 2002 from two overhangs located about 20 m apart, to serve as an outgroup.

One hundred and eighty colonies were genotyped at six microsatellite loci (Craig et al. 2001, note that all analyses, except that done for linkage disequilibrium, used only five loci—see "Results"). Molecular methods duplicated those described for the 2001 samples in Hughes et al. (2005). All PCR products were run at least twice at different concentrations, with PCRs repeated if bands were not clearly sizable. Two colonies from overhang O were dropped from the analysis as no clear product from locus Cd5 could be obtained after three repeat PCRs, giving a final sample size of 178 individuals. Sample size at each overhang is displayed in Table 1.

Genotyping summary statistics

The main focus of this study was the spatial genetic analyses outlined below. To provide a comprehensive data set we also present standard genotype summary measures of linkage disequilibrium (all six loci) and departures from Hardy–Weinberg (HW) expectations (HW exact tests and score tests, locus Cd6 excluded) conducted with the software package GENEPOP (Raymond and Rousset 1995). Caution is required in the interpretation of some of these results as sample sizes between overhangs varied (range 9–50, see Table 1) and assumptions of HW equilibria are probably unsound given the clonal nature of the mating system.

To compare the observed occurrence of repeat multilocus genotypes with theoretical expectations, probability of identity (PI) was used to calculate the match probability, on the basis of allele frequency data and HW expectations, that two unrelated individuals will share a multilocus genotype by chance. PI and the more conservative PI_{sibs} (see Waits et al. 2001), which estimates the probability of identity between sibs and thus allows for the local genetic structure, were calculated with the software package GENALEX 6 (Peakall and Smouse 2006). We also assessed P_{gen}, the likelihood that repeat multilocus genotypes were shared by chance using the "round robin" method of Parks and Werth (1993), and P_{sex}, the probability that individuals sharing the same multilocus genotype were derived from a distinct sexual reproductive event, using the software package GENCLONE (Arnaud-Haond and Belkhir 2007). We also used the permutation methodologies of GENCLONE to visualise whether the power of the microsatellite markers provided a good estimate of the real number of multilocus genotypes present in the sample (Arnaud-Haond et al. 2005; Gregorius 2005).

Spatial genetic analyses

Data classes

Table 1 shows the five classes of data used in the spatial genetic analyses. Only data set #1, the 'full Wembury + Pembrokeshire data set' included animals from the Pembrokeshire outgroup. This outgroup was excluded from spatial autocorrelation analysis because of the vastly different geographic scale of Wembury–Pembrokeshire comparisons. The four remaining Wembury-only data sets were used in all genetic analyses. Data set #2, the 'full Wembury data set', lacked the Pembrokeshire animals but was otherwise the same as data set #1. It is equivalent to a 'ramet level' analysis as defined by Arnaud-Haond and Belkhir (2007). Data set #2 contained 157 individuals of which 90 shared their multilocus microsatellite genotype with at least one other individual. We were very interested in these repeated genotypes as they may have represented multiple offspring from polyembryonous broods (see "Results and discussion"). Removing all 90 repeated genotypes left 67 individuals that contained 'unique genotypes only'—this was data set #5. A less constrained method of

Table 1 Summary of the five data sets used in the genetic analyses

#	Overhang ID	Wembury									Pembrokeshire		n
		G	*H*	*I*	*J*	*K*	*L*	*M*	*N*	*O*	Pem *Y*	Pem *Z*	
1	Full Wembury + Pembrokeshire data set	12	9	19	50	15	9	10	18	15	14	7	178
2	Full Wembury data set	12	9	19	50	15	9	10	18	15	–	–	157
3	One of each genotype	9–12	6–9	13–17	21–25	8	6–7	7–9	12–14	7–11	–	–	97
4	Random $n = 97$	4–9	3–8	10–15	26–35	6–12	3–9	4–9	9–13	7–12	–	–	97
5	Unique genotypes only	9	6	12	10	5	5	6	8	6	–	–	67
	Ng:N	0.75	0.66	0.63	0.20	0.33	0.56	0.60	0.44	0.40			

Values represent numbers of individual colonies within each overhang, totaled in the right hand column. The range of values shown in data sets #3 and #4 reflects differences between randomisations. 'Ng:N' values represent the proportion of unique multilocus genotypes to the number of individuals in each overhang

avoiding repeats was to retain a single representative of each genotype. Owing to the prodigious number of possible combinations of the 90 individuals that shared 30 different microsatellite genotypes, we ran analyses on 20 randomly chosen subsets and called this data set #3 'one of each genotype'. This data set is broadly equivalent to the 'genet level' resampling approach of Arnaud-Haond and Belkhir (2007). Differences in results between #2 'full Wembury data set' and #3, 'one of each genotype' would be expected to result from repeat genotypes and the reduction in sample size ($n = 157$ cf. 97). To better understand the influence of sample size we created a further 20 subsets of $n = 97$, but this time randomly reduced without respect to genotype so that shared microsatellite genotypes remained. This was data set #4 'random $n = 97$'.

Note that the methodology of randomising the data to generate multiple sets of 'one of each genotype' is conservative to situations where sampling is not exhaustive, repeated multilocus genotypes are relatively rare and widespread over the sample area, and/or where there are expectations that the multilocus markers have insufficient power to fully differentiate every clonal individual. This differs from some other methods for differentiating sexual from asexual processes where multiple individuals of the same multilocus genotype are pooled across space to provide a single geographic coordinate and thus a single reduced data set (see e.g. Hämmerli and Reusch 2003; Alberto et al. 2005)—the 'genet level' 'central coordinates approach' of Arnaud-Haond and Belkhir (2007).

Software

The main spatial genetic analyses were performed with the software package GENALEX. Pairwise individual by individual genetic distances were calculated with the method fully explained in Smouse and Peakall (1999). This method produces a matrix of squared dissimilarity distances between individuals for each locus. Values can be summed across loci to produce a single multivariate matrix of pairwise, multilocus genetic distances.

Analysis of molecular variance (AMOVA)

Individual pairwise genetic distance matrices were analysed by AMOVA (Michalakis and Excoffier 1996). The main analyses used the multivariate matrix described above. Tests on individual loci were also performed on the 'full Wembury + Pembrokeshire data set'. Total genetic variation was partitioned into three hierarchical levels: among regions (Wembury vs. Pembrokeshire), populations (rock overhangs) and individuals (single colonies, Peakall et al. 1995). Variation is presented both as the percentage of the total variance and as Φ-statistics (*F*-statistic analogues): Φ_{PT} is the correlation among individuals within populations; Φ_{PR} the correlation among populations within regions; and Φ_{RT} the correlation among regions. F-statistics derived from the alternative input as a codominant allelic distance matrix are provided for comparison with the multiallelic, total data set. Statistical significance was determined by random permutation (always set to 1,000).

Spatial autocorrelation

The main spatial genetic structure analysis used the multivariate matrices described above. This genetic distance matrix was matched as an input to the pairwise geographical distance matrix, calculated as the Euclidean distance between *x*, *y*-coordinates. As we were primarily interested in structure at small geographic scales, discrete distance classes were set on an arithmetic doubling scale (with the final distance class extended slightly). The generated autocorrelation coefficient, *r*, is bounded by −1 and +1 and is closely related to Moran's-I. Tests of statistical significance were performed by both random permutation to provide 95% confidence intervals around the null hypothesis of no spatial genetic structure ($r = 0$), and by bootstrapping to estimate the 95% confidence interval about the *r* value itself (see Peakall et al. 2003). For comparison we repeated analyses using the kinship coefficients of Loiselle et al. (1995) and Ritland (1996). With the software package GENCLONE (Arnaud-Haond and Belkhir 2007) three additional autocorrelation procedures adapted to the existence of replicate genotypes were performed: (1) using a resampling approach to automatically create and analyse subdatasets with each multilocus genotype represented only once (equivalent to that performed manually in the twenty 'one of each genotype' datasets above) to generate 95% confidence intervals of the influence of multilocus genotypes themselves; (2) using a weighted approach to remove the distances among pairs of identical genotypes from the data set; (3) using central coordinates for each replicated multilocus genotype (fully described by Arnaud-Haond and Belkhir 2007).

Mantel tests

Correlation between the genetic and geographic distance matrices was also analysed by Mantel tests of matrix correspondence (Mantel 1967; Peakall et al. 1995).

Results

Genotyping summary statistics

We found identical genotypes at loci Cd5 and Cd6 in 171 out of the 178 *C. denticulata* colonies. This extreme linkage

disequilibrium prompted us to look at the Genbank sequences of the original primer note of Craig et al. (2001) which showed the Cd6 clone to be identical to the Cd5 clone at 491 of the 498 bp. Considerable similarity exists in the published primer sequences, with primer overlaps accounting for the 10 bp difference in the size of PCR products. We conclude that loci Cd5 and Cd6 are actually the same microsatellite and urge future workers to use one or other, but not both, of the primer pairs. All analysis for this study only used the data from Cd5. With the probably unlikely exception that some of the differences between the Cd5 and Cd6 genotypes are due to indels in the different primer flanking regions this duplication acts as an independent, blind scored, estimate of the genotyping error rate. Assuming it improbable that an identical miscoring error is made in both loci in the same individual the error rate for each locus is half that reported above, so ca. 2%. Of the remaining 14 locus pair comparisons linkage disequilibrium may have been detected between loci Cd4 and Cd7 ($\chi^2_{20} = 41.59$, unadjusted $P = 0.003$, sequential Bonferroni 5% $P_{crit} = 0.0036$) as well as Cd7 and Cd8 ($\chi^2_{20} = 32.90$, unadjusted $P = 0.008$, sequential Bonferroni 5% $P_{crit} = 0.0038$).

We observed two significant departures from HW equilibrium out of the 66 possible locus × overhang calculations (Cd7 in overhang K and Cd15 in overhang J—fully tabulated in S3.1 in the ESM). Across all overhangs locus Cd7 showed an overall significant departure from HW expectations. Across all loci overhangs J and K both showed an overall significant departure from HW expectations. None of the loci showed significant positive F_{IS} values across all overhangs suggesting that the presence of null alleles was negligible. Score tests (Rousset and Raymond 1995) for heterozygote deficiencies reported a strong deficit in locus Cd15, which was probably caused by three individuals being incorrectly scored as homozygous for a unique allele due to a sizing error. Score tests showed that no loci or overhangs had a heterozygote excess.

Permutation methodologies that produced plots of the increase in detected multilocus genotypes with increasing number of loci did not show an asymptote, suggesting that we overestimated the true number of repeated multilocus genotypes. Across all colonies the probability of identity (PI = 0.008), PI_{sibs} (0.110) and P_{gen} (2 colonies $P > 0.05$; 48 colonies $P = 0.05$–0.01; 21 colonies $P = 0.01$–0.005) measures confirmed the relatively low power of the five microsatellite loci to differentiate genetically distinct individuals, although given the expected violation of HW principals, caution is required in the exact interpretation of these results. A slightly more conservative version of one of these tests that takes into account the estimated F_{IS} in the population (Young et al. 2002), '$P_{gen}(f)$' slightly increased the probabilities (7 colonies $P > 0.05$; 54 colonies $P = 0.05$–0.01; 21 colonies $P = 0.01$–0.005) suggesting that HW deviations were not conservative to the initial results. With the sample size of 178 individuals the PI values predict that 1.4 (PI) and 19.5 (PI_{sibs}) individuals would share the same multilocus genotype by chance alone. This is clearly many fewer than the 90 colonies found in this study with shared multilocus genotypes and suggests that the multiple settlement and survival of polyembryonous clone mates can be detected but that our measures are imprecise. Similarly the P_{gen} and $P_{gen}(f)$ derived binomial measures P_{sex} and $P_{sex}(f)$ suggest that 22 or 23, respectively, of the 30 distinct multilocus genotypes that were found in more than one colony could statistically have resulted from sexual rather than asexual processes, although the low power of this test for the current data set where many repeat genotypes are found only a few times (16 of the 30 repeated multilocus genotypes were only found twice) must be considered.

S3.2 and S3.3 in the ESM tabulate allele frequencies for each overhang and sample sizes, number of alleles, number of effective alleles, information index, observed and expected heterozygosity and fixation index for each overhang × locus combination.

Spatial genetic analyses

AMOVA

Table 2 summarises the AMOVA results. Note that the majority of variation is distributed within individual rock overhangs (populations). Table 3 shows the pairwise Φ_{PT} values. The inset of Table 3 shows the multi-dimensional relationship between all populations by Multi-Dimensional-Scaling (software package PRIMER; Clarke and Gorley 2001) indicating the lack of differentiation between populations G, H, I and J.

With only the 'full Wembury data set', AMOVA revealed a Φ_{PT} of 0.1998, with 80% of the variation distributed within populations and 20% among populations (also shown in Table 2). This Φ_{PT} was greater than any of the 20 'one of each genotype' subsets that had replicate microsatellite genotypes removed (Mean $\Phi_{PT} = 0.1621$, SD = 0.0090, $n = 97$). In comparison to the 'full Wembury data set' more variation was found within populations (range 83–87%) and less among populations (13–17%) in the 'one of each genotype'. These effects appear not to be due to the reduced sample size, as the 'random $n = 97$' subsets showed Φ_{PT} values similar to that of the 'full Wembury data set' (mean = 0.1972, SD = 0.0210), and significantly greater than the 'one of each genotype' subsets (Mann–Whitney $W = 652.5$, $P < 0.0001$). The 'random $n = 97$'subsets also had variation within (76–83%) and among (17–24%) populations similar to that of the 'full Wembury data set'.

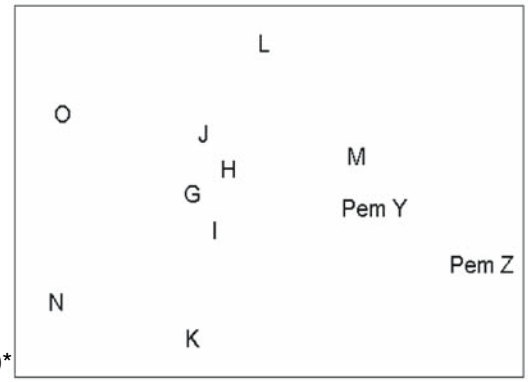
Table 2 Results of AMOVA showing Phi values and the percentage of variation apportioned among regions (Wembury versus Pembrokeshire), populations (rock overhangs) and individuals for individual microsatellite loci and the multivariate total in the "Full Wembury + Pembrokeshire data set", $n = 178$. Figures in parenthesis are equivalent values for the alternative input as codominant allelic distance matrix for calculation of F_{st}. Figures in square brackets show the percentage of variation apportioned among populations and individuals in the "Full Wembury data set", $n = 157$ to show the similarity of signal in all five loci when Pembrokeshire samples are removed from the analysis

	Total, %	Cd4, %	Cd5, %	Cd7, %	Cd8, %	Cd15, %
n Alleles	25	2	5	10	3	5
Among regions	17 (10)	0	0	41	2	0
Among pops./regions	16 (10) [20]	22 [25]	21 [24]	10 [16]	8 [9]	28 [29]
Indiv./within pops	67 (80) [80]	78 [75]	79 [76]	49 [84]	90 [91]	72 [71]
Φ_{RT}	0.166 ($F_{rt} = 0.010$)***	-0.102	−0.107	0.407***	0.016	-0.123
Φ_{PR}	0.189 ($F_{sr} = 0.106$)***	0.223***	0.209***	0.163***	0.086**	0.270***
Φ_{PT}	0.323 ($F_{st} = 0.195$)***	0.143*	0.125*	0.504***	0.010*	0.180***

P values represented by asterisks = * 0.050–0.011, ** 0.010–0.006, *** 0.005–0.001

Table 3 Pairwise Φ_{PT} values plus significance asterisks (see Table 2) of full data set

	G	H	I	J	K	L	M	N	O	Pem Y
G										
H	0.012									
I	0.000	0.032								
J	0.056	0.022	0.062*							
K	0.123*	0.228***	0.059	0.281***						
L	0.112*	0.175*	0.236***	0.244***	0.295***					
M	0.133*	0.157***	0.210***	0.208***	0.313***	0.120*				
N	0.115*	0.321***	0.176***	0.321***	0.239***	0.379***	0.329*			
O	0.064	0.263***	0.208***	0.247***	0.287***	0.259***	0.335***	0.250***		
Pem Y	0.202**	0.202***	0.195***	0.223***	0.291***	0.233***	0.047 7	0.373***	0.418***	
Pem Z	0.353**	0.410***	0.291***	0.340***	0.359***	0.431***	0.229***	0.491***	0.554***	0.00



'*G*' to '*O*' are the individual Wembury overhangs, 'Pem *Y*' and 'Pem *Z*' are the two overhangs of the Pembrokeshire outgroup. Some caution is required in interpreting these pairwise overhang comparisons as sample size varied as described in Table 1. Inset shows an MDS plot of this data set, stress = 0.09. S2B of the ESM provides an aerial photograph of the study site to show the geographic relationship between these overhangs

AMOVA of the 'unique genotypes only' ($n = 67$) exaggerated the trends revealed by the 'one of each genotype' subsets with a low Φ_{PT} of 0.1428, and high variation within (86%) rather than between (14%) populations.

Spatial autocorrelation

Figure 1 shows correlograms of pairwise genetic correlation as a function of geographical distance for the four Wembury data sets (#2–5 in Table 1). All within-overhang pairwise geographic distances were less than 4 m. The 1–2 m distance class also contained some of the between-overhang comparisons of overhangs L and M (hereafter LM). The 2–4 m class contained the remainder of LM as well as some of NN and JJ. The 4–8 m class held only GH and HI, the 8–16 m class only GI and JK. The 16–32 m class contained KL, KM, LN, and MN. The 32–64 m class comprised GJ, GK, HJ, HK, IJ, IK, JL, JM, JN, KM and NO (fully tabulated in S4 of the ESM).

All four data sets had the highest positive *r* values at the smallest distance class of ≤0.125 m. Most values remained positive and significant for distances up to 1 m. Only in the 'full Wembury data set' did *r* values remain consistently positive and significant up to 4 m, although this trend was evident in many of the 'random $n = 97$' runs. The vast majority of the 'one of each genotype' runs had *r* values around zero in the 1–2 m distance class although most runs regained positive, significant autocorrelation for the 2–4 m class. Even though repeat genotypes made up a greater proportion of pairwise comparisons at other within-overhang

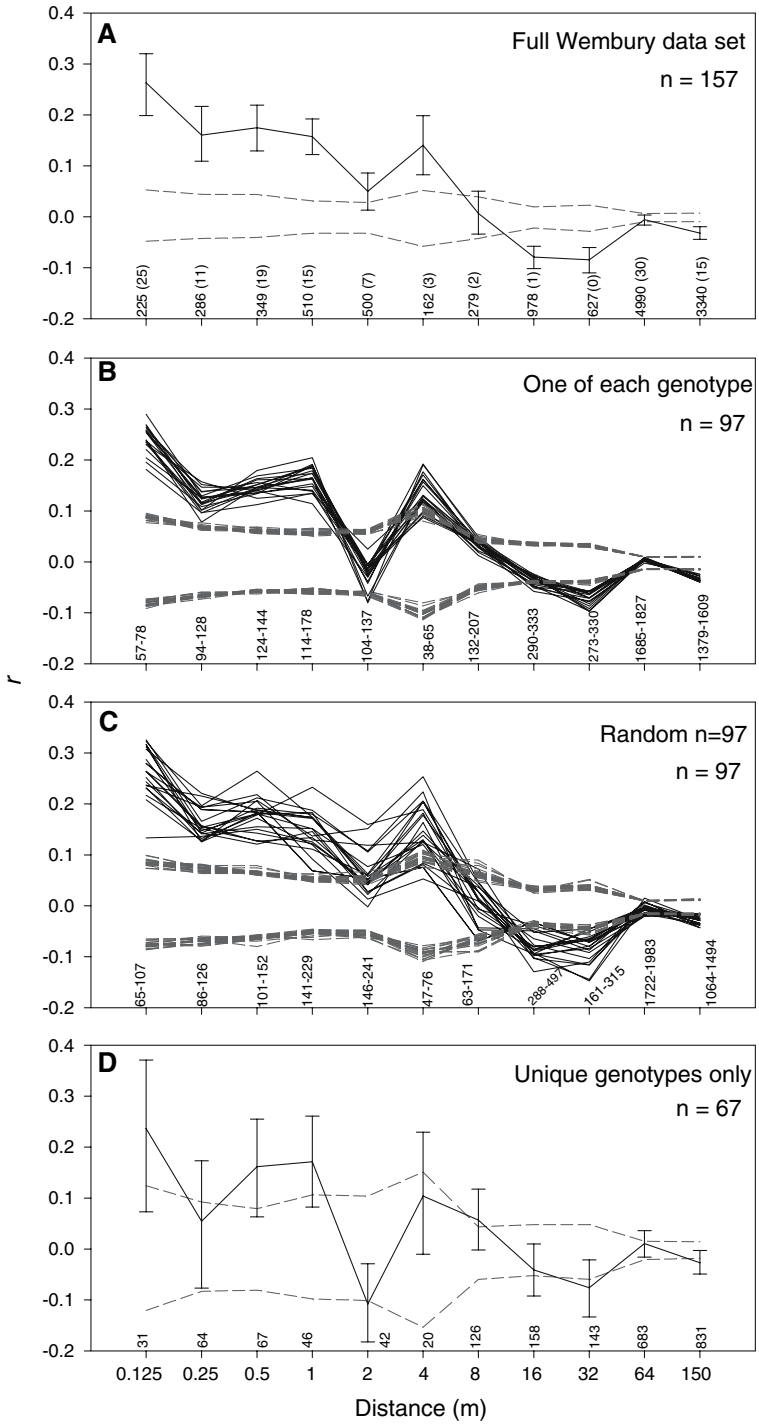


Fig. 1 Correlograms showing the pairwise genetic correlation (*r*, *solid line*) as a function of discrete geographic distance (X-axis, value represents the upper end of the range). **a**–**d** represent the four data classes of Wembury individuals. Only **a** and **c** contain repeat microsatellite genotypes (see "Methods" and Table 1). *Dashed lines* show the 95% confidence interval around the null hypothesis of a random distribution of genotypes as generated by permutation. Error bars are the 95% confidence interval about *r* as estimated by bootstrapping (not shown in **b** and **c** for clarity). *Numbers* of paired individuals at each distance class are provided just above the X-axis. *Value in parenthesis* in **a** is the number of comparisons between repeat genotypes

spatial scales (Fig. 1a, sample size data) the test statistic of genetic similarity changed slightly at 0.125–0.25 m and considerably at the 1–2 m distance class. At intermediate distances between 4 and 32 m autocorrelation decayed such that all data sets displayed significant negative autocorrelation at the 16–32 m class. In the between-overhang comparisons of 4–16 m, omission of repeat genotypes slightly increased genetic similarity. This counterintuitive result likely stems from the dominance of comparisons between overhangs G, H and I at these spatial scales. G, H and I were found to be genetically uniform by AMOVA (Table 3) and are composed of a relatively low proportion of repeated genotypes (33% compared to 66% in the remaining overhangs combined). All data sets showed autocorrelation around zero at 32–64 m, probably due to the clustering of genetically uniform overhangs GJ, HJ and IK into otherwise dissimilar pairwise comparisons. Significantly negative autocorrelation was always seen for the

largest distance class (64–150 m). Repeating analyses using the kinship coefficients of Loiselle et al. (1995) and Ritland (1996) and the programme GENCLONE gave broadly similar results for the 'Full Wembury data set' (='ramet level' analysis, Fig. 2). The greater statistical power of GENCLONE's resampling approach allowed small but significant impacts of repeat genotypes on genetic structuring to be also detected at 0.125–0.5, 1–2 and 4–16 m (Fig. 2) that were in some instances not so apparent in the main analyses shown in Fig. 1. Using GENCLONE's weighted approach to remove the distances among pairs of identical genotypes from the data set allows for an estimation of the clonal subrange, the distance beyond which repeat multilocus genotypes have negligible effects on genetic structure as less than 1% of pairs are of the same genotype (Alberto et al. 2005). As shown in S5 of the ESM this distance class, where correlograms merge, is at 8–16 m, a distance that represents the lower end of the inter-overhang comparisons. As predicted in the "Materials and methods" section, using central coordinates for each replicated multilocus genotype gave a pattern that fluctuated wildly and is difficult to interpret (S5 of the ESM).

As the ability to detect nonrandom genetic structure is influenced by the distance class size chosen, we also analysed increasing cumulative distance size classes from the minimum resolution used in this study of 0–0.05 m up to 0–120 m (maximum individual to individual distance in the data = 133 m). Thus the analysis displayed in Figs. 1 and 2 used discrete distance classes (e.g. 0–1, 1–2, 2–3), whereas the second analysis, displayed in S6 of the ESM, used cumulative distance classes (e.g. 0–1, 0–2, 0–3). The highest value of *r* was found at the smallest distance class of 0–0.05 m. As cumulative distance increased the *r* value dropped, indicating a loss of resolution. Negative autocorrelation at greatest pairwise geographic distances (against which the statistical comparison would be made), combined with strong positive autocorrelation at the intra-overhang scale, helped *r* values remain positive at large cumulative distances.

Mantel tests

Results with the 'full Wembury + Pembrokeshire data set' ($rxy = 0.137$, $P = 0.007$) and the 'full Wembury data set' ($rxy = 0.073$, $P = 0.021$) corroborated spatial autocorrelation data in indicating that genetic structuring was present. When sample sizes were reduced in data sets #3–5, however, the less powerful Mantel tests generally failed to detect structuring. The lack of resolution of the Mantel tests probably results from a more complex relationship between geographic and genetic distances than the linear assumptions of Mantel procedures (as represented by the inconsistent slope of the correlograms in Figs. 1 and 2, full results in S7 of the ESM).

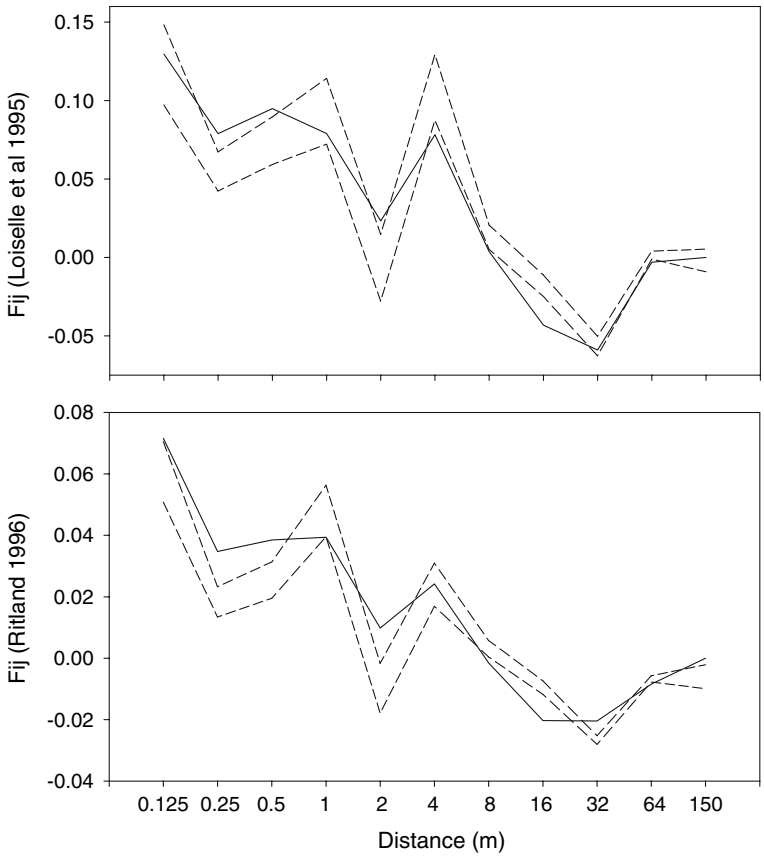


Fig. 2 Correlograms generated using the kinship coefficients of Loiselle et al. (1995, *upper figure*) and Ritland (1996, *lower figure*). *Solid lines* represent results for the 'Full Wembury data set' (='ramet level' analysis). *Dashed lines* show the 95% confidence intervals generated by the resampling approach of GENCLONE that shows structure in the absence of multilocus genotypes

Discussion and conclusions

The discrete distribution of *Crisia denticulata* made it possible to define individual rock overhangs as testable subpopulations a priori. In doing so AMOVA showed significant differentiation between most rock overhangs separated by only a few meters. Spatial autocorrelation statistics, which do not require subpopulations to be defined a priori, also revealed significant genetic dissimilarity at scales representing between-overhang pairwise comparisons, with genetic similarity found within overhangs. Spatial autocorrelation techniques have been used extensively by botanists (e.g. Hämmerli and Reusch 2003; Vekemans and Hardy 2004; Migliaccio et al. 2005), but have limited history in studies of sessile marine animals where results have (Yund and O'Neil 2000) or have not (McFadden and Aydin 1996) found moderately small-scale genetic structuring. The 4–8 m distance class at which spatial autocorrelation changed from being positive to negative, which represents the extent of a genetically similar patch, is remarkably similar to the 5.4–7.2 m distance class described by Yund and O'Neil (2000) in a population of the non-polyembryonous, spermcast colonial ascidian *Botryllus schlosseri*. Spatial autocorrelation statistics are particularly useful for the analysis of small-scale genetic structure because they allow structure to be detected down to the minimum distance between sampled individuals. In our study fine scale analysis revealed the strongest genetic similarity at the smallest pairwise distances (same or adjacent 5 × 5 cm quadrat division), suggesting restricted gene flow within overhangs that would not have been detected by traditional methods. Such a result suggests small-scale dispersal of sperm and/or larvae in this species.

By analysing data with and without putative multiple representatives of individual clones, components of genetic structure attributable to asexual processes can be separated from the effects of isolation-by-distance during sexual reproduction. In this study the strength of within-overhang genetic similarity was increased by a small but significant amount by the presence of repeat multilocus genotypes at the distance classes of 0.125–0.5, 1–2 and 4–16 m. As demonstrated by the PI tests, permutation methodologies, P_{gen} and P_{sex} tests repeat genotypes are expected even in the absence of cloning, their probability being a function of relatedness and the frequency distribution of alleles at the loci studied. Although conservative to the conclusions drawn, it is almost certain that a proportion of the repeated genotypes in our data were not polyembryonous clone mates. Thus, although 0.125–0.5, 1–2 and 4–16 m may represent statistically prominent distances of cloned larval dispersal, we cannot exclude the possibility that some repeat genotypes are genetically similar individuals (e.g. full- or half-siblings) that our markers cannot distinguish rather than polyembryonous clonemates. In that case these distances could reflect the scale of non-clonal larval dispersal (same maternal colony but different gonozooids) and perhaps the movement of fertilising sperm. Direct tests of sperm and larval transport under field conditions would be needed to investigate this (e.g. Levin 1990). Our findings, that embryonic cloning makes a detectable (Φ_{PT} values were reduced when shared genotypes were removed from the dataset) but minor contribution to the genetic structuring of a sessile polyembryonous species, is consistent with previous reports from a terrestrial pine (Rogers et al. 1999) and a red alga (Engel et al. 2004, where repeated diploid genotypes could also result from independent fertilisations between haploid parents, further weakening the role that polyembryony plays in determining the genetic structure). The genetic footprint of this form of polyembryony therefore differs greatly from the vegetative spread through rhizomes or plant fragments reported for clonal marine plants where autocorrelation studies must explicitly consider the fact that individual clones can dominate large areas (e.g. Montalvo et al. 1997; Reusch et al. 1999; Hämmerli and Reusch 2003).

Despite the finding of genetic similarity at small spatial scales, AMOVA reported the majority of genetic variation (67 or 80% depending on the data class used) to be distributed between colonies within individual rock overhangs. Similar findings have been reported for a polyembryonous intertidal red alga (Engel et al. 2004). Despite finding that the spatially closest individuals had the highest pairwise genetic similarity such results suggest that closely situated potential mates contain a significant proportion of the available genetic diversity within a population. This runs counter to Ryland's (1996) suggested explanation of polyembryony in cyclostome bryozoans that the relative loss of offspring genetic diversity caused by polyembryony is reduced in viscous, locally homogeneous, populations. What exactly constitutes 'sufficient' local genetic variation to favour a purely sexual strategy over the polyembryonous alternative is, however, difficult to know, which is a weakness of this study and a challenge for the testing of Ryland's hypothesis itself. It is clear that we are a long way from being able to quantify the precise costs of polyembryony against the alternatives of a purely sexual or asexual mode of reproduction in species such as cyclostome bryozoans. Given the complexity of the focused attempts over the last few decades to quantify the relative costs of apparently 'simple' sexual versus asexual reproduction (e.g. Bell 1982; Goddard et al. 2005) this task seems daunting.

Larval and post-settlement mortality are thought to be the main factors limiting population growth in most marine organisms (e.g. Morgan 1995), although studies of brooded lecithotrophic larvae, such as found in the Bryozoa, are limited. If much of a mother's fitness depends on the sheer

number of larvae that she is able to produce and larval mortality occurs randomly with respect to larval genotype (but see Schmidt and Rand 1999), then the apparent costs of polyembryony may be invisible to selection. Genetic diversity of a female's offspring may be favoured, however, if genetic diversity partially reduces brood-wide larval mortality by increasing the chance of genotype-environmental matching (the 'tangled bank' hypothesis of Bell 1982). Although polyembryony in *C. denticulata* forces genetic uniformity within a single brood, some genetic diversity is maintained by the presence of multiple broods (Hughes et al. 2005). This diversity may be sufficient to accrue much of the larval survival benefit. Further, the sequential release of genetically uniform embryos over an extended time period by cyclostomes and red algae seems to allow one larval genotype to be repeatedly tested against a variety of environments as conditions change over the time period of brood release. Such testing of a few genotypes against a changing environment may differ very little from regular sexual reproduction that tests many genotypes against an environment at once. This type of polyembryony that spreads the cloned genet across space and time with a concurrent retention of genetic diversity between broods perhaps seems less of a paradox than initially thought.

A summary of the different hypotheses, tests and results of this study are tabulated as S8 of the ESM.

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